FEBS Letters 549 (2003) 72–76

FEBS 27496

Different mechanisms are used by insulin to repress three genes that contain a homologous thymine-rich insulin response element

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Received 3 February 2003; revised 19 May 2003; accepted 30 June 2003

First published online 17 July 2003

Edited by Robert Barouki

Abstract Insulin rapidly and completely inhibits expression of the hepatic insulin-like growth factor binding protein-1 (IGFBP-1), phospho*enol*pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) genes. This inhibition is mediated through a phosphatidyl inositol 3-kinase-dependent regulation of a DNA element, termed the thymine-rich insulin response element, found within the promoters of each of these genes. This has led to the conclusion that these three promoters are regulated by insulin using the same molecular mechanism. However, we recently found that the regulation of the IGFBP1 but not the PEPCK or G6Pase genes by insulin was sensitive to rapamycin, an inhibitor of mTOR. Here, we present further evidence that different regulatory pathways mediate the insulin regulation of these promoters. Importantly, we identify a protein phosphatase activity in the pathway connecting mTOR to the IGFBP-1 promoter. These data have major implications for the development of molecular therapeutics for the treatment of insulin-resistant states such as diabetes and hypertension.

Key words: Protein phosphatase; Insulin; IGFBP-1; G6Pase; mTOR

1. Introduction

Insulin is secreted from the pancreatic β-cells in response to increasing glucose levels in the blood. The result is the restoration of plasma glucose levels and conversion of excess glucose to glycogen [1]. Insulin achieves this through the regulation of protein activity, localisation and expression. Indeed, insulin is known to regulate the expression of over 100 gene products in multiple cell types [2]. For example, insulin completely inhibits the expression of hepatic insulin-like growth factor binding protein-1 (IGFBP-1), phospho*enol*pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). IGFBP-1 binds insulin-like growth factors (for review see [3]), while PEPCK and G6Pase are rate-controlling enzymes of hepatic gluconeogenesis [4,5]. All of these genes are over-expressed in diabetes [6–9]. Meanwhile, PEPCK over-expression in animals promotes hyperglycemia, impaired glucose tolerance and insulin resistance [10–12], and increased G6Pase expression in mice produces a metabolic profile similar to Type 2 diabetes [13]. Interestingly, these three gene promoters contain a homologous DNA element, termed the thymine-rich insulin response element (TIRE), through which insulin mediates at least part of its inhibitory effect [5]. The presence of this element in these promoters initially suggested that insulin employs a common mechanism to repress each. This hypothesis was strengthened when several groups observed that insulin requires phosphatidyl inositol 3-kinase (PI 3-kinase) activity for regulation of PEPCK [14,15], G6Pase [16] and IGFBP-1 [17] expression. However, the insulin regulation of the IGFBP-1 but not G6Pase or PEPCK gene promoters is sensitive to rapamycin [14,17,18]. Therefore, the activity of mTOR is required for regulation of IGFBP-1 but not PEPCK or G6Pase by insulin. It is not yet understood how mTOR regulates the IGFBP-1 promoter but studies in yeast have implicated Tor (yeast mTOR) in the regulation of a protein phosphatase [19,20]. Therefore, we investigated the effect of okadaic acid, a diarrhoetic shellfish toxin that selectively inhibits protein phosphatases 1 (PP1) and 2A (PP2A), on the regulation of IGFBP-1 expression. This agent is known to repress PEPCK gene expression [21]; therefore we also examined its effect on the expression of a third TIRE-containing gene, G6Pase. The data presented herein provide further evidence that insulin regulates IGFBP-1 gene expression by a mechanism distinct from that used to regulate G6Pase and PEPCK, and implicates a protein phosphatase in the pathway from mTOR to the IGFBP-1 promoter.

2. Materials and methods

Radioisotopes were obtained from Amersham, Bucks, UK ([γ^{32}P]ATP) and ICN, Thame, Oxfordshire, UK ([α^{32}P]UTP). Insulin was purchased from Novo Nordisk (Crawley, West Sussex, UK), okadaic acid from Calbiochem (Nottingham, UK), and the RNase Protection Assay kit II from AMS Biotech/Ambion (Austin, TX, USA). All other chemicals were of the highest grade available.

2.1. Cell culture

The rat hepatoma cell line H4IIE was maintained in Dulbecco's Modified Eagle's medium (DMEM) containing 1000 mg/l glucose, 5% (v/v) foetal calf serum, as described previously [22]. Cells were incubated with hormones, at 37°C, for the times and at the concentrations indicated in the figure legends.

2.2. RNA isolation and RNase protection assay

H4IIE cells were serum-starved overnight and treated with hormone/inhibitor for the times and at the concentrations indicated. To-

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Abbreviations: G6Pase, glucose-6-phosphatase; IGFBP-1, insulin-like growth factor binding protein-1; PI 3-kinase, phosphatidyl inositol 3-kinase; TIRE, thymine-rich insulin response element; PKB, protein kinase B; PEPCK, phospho*enol*pyruvate carboxykinase; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A

0014-5793/03/$22.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00774-9

tal cellular RNA was isolated using TriReagent™ (Sigma) following the manufacturer's instructions. An RNase protection assay (RPA) was performed to determine the relative amounts of IGFBP-1, G6Pase and cyclophilin mRNA in each sample [22,23]. Band intensity was quantified on a phosphorimager (Fuji) and data calculated as a ratio of IGFBP-1 or G6Pase to cyclophilin mRNA.

2.3. Preparation of cell extract for kinase assays and Western blot

H4IIE cells were incubated in serum-free medium with hormones and inhibitors for the times and at the concentrations indicated. Cells were then scraped into ice-cold lysis buffer (25 mM Tris–HCl, pH 7.4, 50 mM NaF, 100 mM NaCl, 1 mM sodium vanadate, 5 mM EGTA, 1 mM EDTA, 1% (v/v) Triton X-100, 10 mM sodium pyrophosphate, 1 mM benzamidine, 0.1 mM PMSF, 0.27 M sucrose, 2 μM microcystin and 0.1% (v/v) 2-mercaptoethanol). Cell debris was removed by centrifugation at 13 000 × *g* and the protein concentration determined. Protein kinases were immunoprecipitated and assayed as described previously [18].

3. Results and discussion

3.1. Insulin requires the activity of a phosphatase to repress IGFBP-1

Okadaic acid is a potent inhibitor of PP1 and PP2A [24,25]. Previous studies suggested that okadaic acid could mimic the effect of insulin on PEPCK gene expression, implicating protein phosphorylation as a major mechanism by which insulin regulated this gene promoter [21]. In contrast, treatment of H4IIE cells with okadaic acid alone, even at concentrations up to 1 μM, has little effect on IGFBP-1 mRNA levels, but the presence of this agent dramatically reduces insulin repression of IGFBP-1 (Fig. 1A). Okadaic acid antagonises the insulin regulation of both basal and dexamethasone-induced IGFBP-1 expression (Fig. 1A), although the effect of okadaic acid is not as complete when the gene is induced. In addition, within the same experiment, the presence of 1 μM okadaic acid reduces G6Pase gene expression, similar to its reported effect on PEPCK expression [21]. However, the repression is not as complete as that observed in the presence of insulin (Fig. 1B). Meanwhile, lower concentrations (0.1 μM) of okadaic acid have no significant effect on insulin regulation of IGFBP-1 or G6Pase expression (data not shown). Similarly, insulin regulation of IGFBP-1 gene expression is not significantly reduced by the PP1 inhibitor tautomycin (data not shown). Unfortunately, a third phosphatase inhibitor, Calyculin-A, is extremely toxic to the H4IIE cells at concentrations required to inhibit PP2A; therefore we were not able to make use of this inhibitor.

These data confirm the importance of phosphorylation in the regulation of both promoters and although they suggest the involvement of a phosphatase in insulin regulation of the IGFBP-1 promoter, they do not reveal the identity of the phosphatase involved. We have previously shown that mTOR is required for the regulation of IGFBP-1 gene expression by insulin [18]. Interestingly, an inhibitor of mTOR only partially reduces insulin regulation of IGFBP-1 expression, reminiscent of the effect of okadaic acid. In mammals, relatively little is known about the role of mTOR as a direct transcriptional regulator. However, in yeast, Tor is a regulator of several transcription factors [26,27]. Genetic evidence suggests that proteins such as Tap42p, Ure2 and Bmh2p are important in the Tor regulation of specific transcription factors [20,27]. Tap42p is a protein that binds and inhibits the protein phosphatase SIT4. Upon Tor inactivation by rapamycin treatment or starvation, SIT4 is released from Tap42,

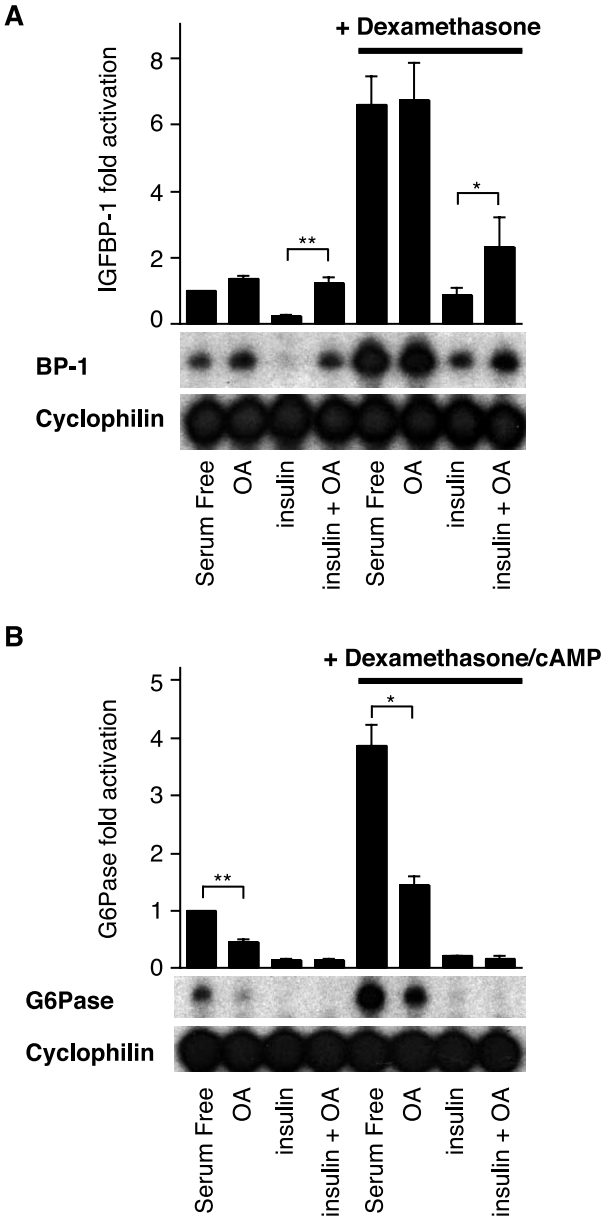

Fig. 1. Treatment of H4IIE cells with okadaic acid has opposing effects on insulin regulation of IGFBP-1 and G6Pase gene expression. H4IIE cells were serum-starved overnight prior to a 3-h incubation with dexamethasone (500 nM), cAMP (8CPT-cAMP, 0.1 mM), insulin (10 nM), or okadaic acid (OA, 1 μM) as indicated. Cells were lysed, total cellular RNA isolated, and an RPA performed to assess A: IGFBP-1 (BP-1) and B: G6Pase mRNA levels. Results are presented as fold induction relative to control (serum-free) and are means ± S.E.M. of two experiments performed in duplicate (upper panels). Representative experiments (lower panels) are also shown. $^{*}P < 0.05$; $^{**}P < 0.01$.

leading to the dephosphorylation and nuclear translocation of Gln3 and subsequent activation of target genes [19,20,28]. Therefore, in yeast Tor appears to negatively regulate a phosphatase. Human α4 protein is the closest mammalian homologue of Tap42 [29,30]. α4 can bind to the protein phosphatases PP2A, PP4 and PP6; however, whether the formation of this complex is rapamycin-sensitive remains controversial. Meanwhile, mTOR has also been proposed to directly inhibit PP2A, thereby providing a mechanism by which phosphorylation is maintained on molecules such as ribosomal protein

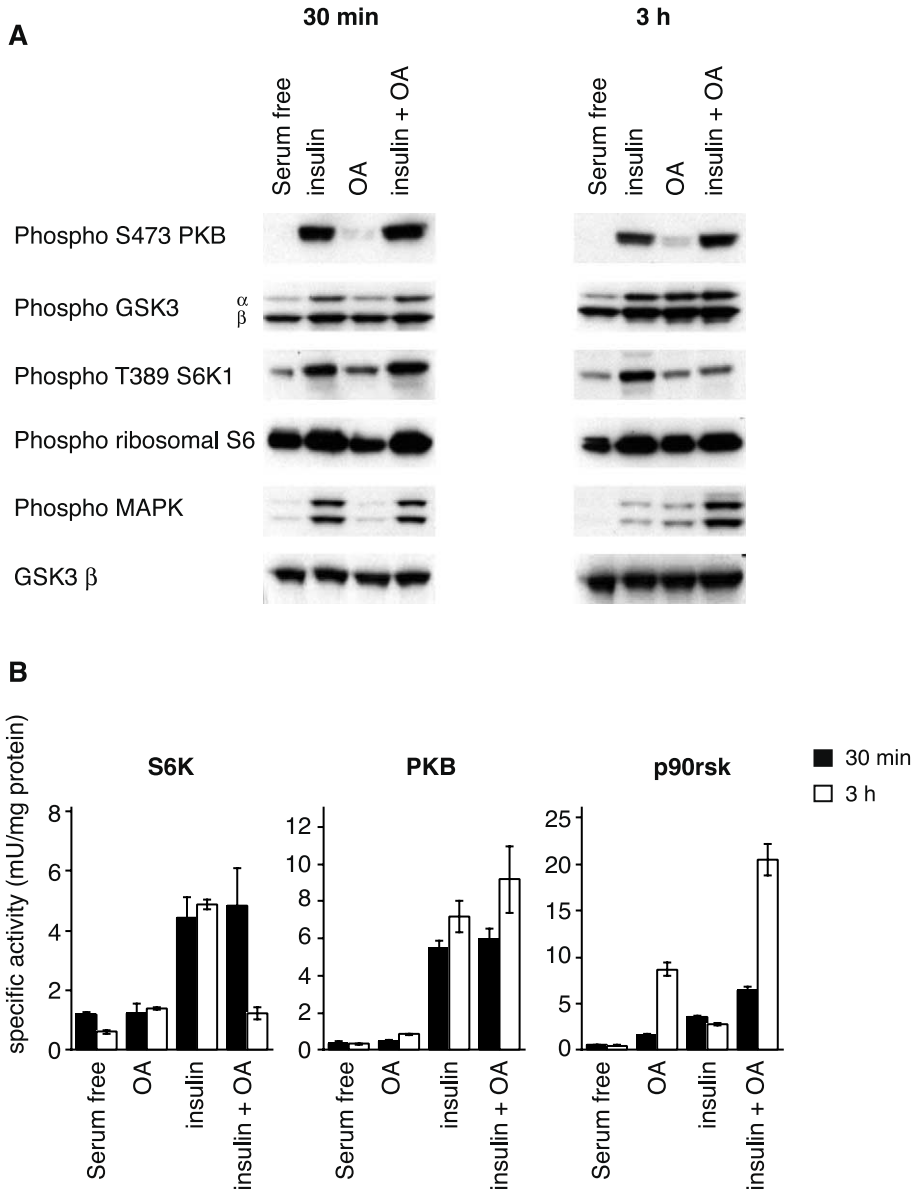

Fig. 2. Effect of okadaic acid on insulin signalling in H4IIE cells. H4IIE cells were serum-starved overnight and incubated with insulin (10 nM) and/or okadaic acid (OA, 1 μM) for the times indicated. A: Cells were lysed and the protein lysates immunoblotted with antibodies as labelled (phospho; phosphospecific to Thr-202 and Tyr-204 of p42/p44 MAPK or Ser-21/9 (GSK3α/β)). Similar results were obtained from two experiments. B: Alternatively, S6K, PKB and p90rsk were immunoprecipitated and specific activity assayed. Results are means ± S.E.M. of two experiments performed in triplicate.

S6 kinase (S6K1), 4EBP-1 [31,32] and PKCδ [33]. In contrast, our data suggest that insulin signalling in liver requires an okadaic acid-sensitive phosphatase activity for regulation of IGFBP-1 (Fig. 1A). If this is true then incubation of cells with okadaic acid should affect signalling pathways that lie between mTOR and the IGFBP-1 gene promoter.

3.2. *Effect of okadaic acid on insulin signalling pathways in H4IIE cells*

Treatment of H4IIE cells with 1 μM okadaic acid for 30 min does not significantly alter insulin regulation of S6K, its substrate ribosomal protein S6 (both downstream of mTOR), or the other insulin signalling molecules, protein kinase B (PKB), GSK3 (a target of PKB), or p42/p44 MAPK (Fig. 2). However, after a 3-h treatment, insulin activation of S6K was significantly reduced, while activation of the p42/44 MAPK pathway was induced (Figs. 2 and 3). This is similar to the effect of okadaic acid in adipocytes [34]. Interestingly, the phosphorylation of GSK3β (Ser-9) increases with okadaic acid treatment, in the absence of any activation of PKB (Fig. 2). Therefore, it is likely that okadaic acid promotes the phosphorylation of GSK3 through activation of p90RSK, rather than through the activation of PKB. p90RSK will phosphorylate and inhibit GSK3 in vitro [35,36] and in intact cells [37].

We have previously found that agents that strongly activate the p42/p44 MAPK pathway can reduce the sensitivity of the IGFBP-1 (but not PEPCK or G6Pase) promoter to insulin [23]. Therefore, it was possible that okadaic acid exerts its effect on IGFBP-1 expression through its ability to strongly

induce p42/p44 MAPK (Figs. 2 and 3). However, treatment with a selective inhibitor of the p42/p44 MAPK pathway does not affect the action of okadaic acid on IGFBP-1, at concentrations that fully block insulin and okadaic acid activation of p42/44 MAPK (Fig. 3B). It seems more likely that okadaic acid is exerting its effects on IGFBP-1 gene expression through the down-regulation of the mTOR pathway (Fig. 2). This is consistent with our findings that reduction of mTOR signalling, by rapamycin or removal of amino acids, antagonises the repression of IGFBP-1 gene transcription by insulin [18]. Therefore, we propose that in liver cells mTOR requires a phosphatase activity (possibly PP1 or PP2A-like) to be present in order to activate S6K as well as repress IGFBP-1 gene expression. The identity and direct substrate of the phosphatase is not yet known, but is the subject of ongoing research.

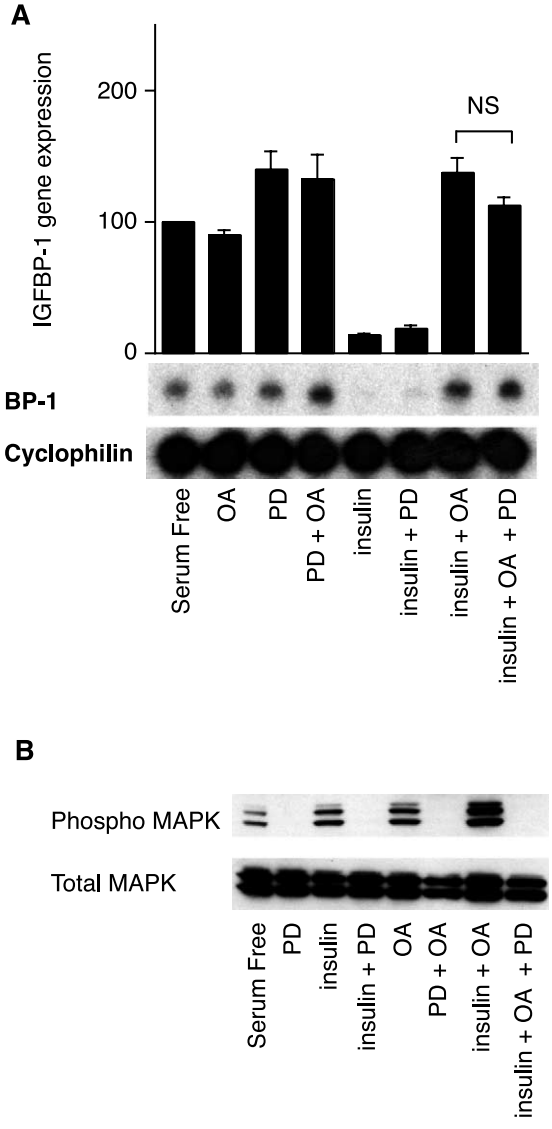

Fig. 3. Regulation of IGFBP-1 gene expression by okadaic acid does not require the activation of the p42/p44 MAPK pathway. H4IIE cells were serum-starved overnight and pre-incubated ±PD184352 (PD) for 30 min prior to a 3-h incubation with insulin (10 nM) and/or okadaic acid (OA, 1 μM) ±PD (2 μM). A: Cells were lysed, total cellular RNA isolated, and an RPA performed to assess IGFBP-1 (BP-1) mRNA levels. Results are means ± S.E.M. of two experiments performed in duplicate. A representative experiment (lower panel) is also shown. NS, not significant. B: Alternatively, cells were lysed and the protein lysates were immunoblotted with antibodies as labelled (phospho; phosphospecific to Thr-202 and Tyr-204 of p42/p44 MAPK). Similar results were obtained in two experiments.

3.3. *Insulin uses distinct mechanisms to regulate TIRE-containing gene promoters*

The treatment of H4IIE cells with phorbol esters [23], hydrogen peroxide [38], or okadaic acid (Fig. 1) blocks (at least in part) the repression of IGFBP-1 gene expression by insulin. Conversely, each of these agents mimics insulin regulation of the PEPCK and G6Pase gene promoters ([21,23,38–40] and C. Sutherland, unpublished data; Fig. 1B). Taken together with the distinct rapamycin sensitivities of these promoters [18], this demonstrates that despite each promoter containing related DNA sequences, the signalling pathways that connect PI 3-kinase activation to each are distinct. Indeed, closer examination of each promoter structure reveals major differences in the context of the distinct TIREs. For example, the PEPCK promoter contains a single TIRE, the G6Pase has three, and the IGFBP-1 has two that lie in an inverted palindrome [5]. In addition, the PEPCK promoter contains a second insulin regulatory sequence, the G6Pase promoter contains an insulin accessory element, while no other insulin regulated DNA elements have as yet been characterised for the IGFBP-1 promoter (although our data have suggested the existence of at least one other insulin response element in this promoter [18]). Therefore, it is perhaps not surprising that distinct mechanisms and probably different DNA-binding proteins are involved in insulin regulation of these promoters.

Finally, insulin resistance underlies the development of several common human disorders, such as Type 2 diabetes mellitus and hypertension [1]. These disorders are on the increase and represent a huge drain on health care expenditure [41]. Current therapies to overcome insulin resistance are relatively ineffectual, therefore a great deal of effort is going into the development of agents that can increase insulin sensitivity or mimic the cellular actions of insulin [42]. The findings presented here demonstrate that care must be taken when designing signalling mimetics, as activation of some insulin signalling pathways can occur simultaneously with inhibition of others. Ideally, a common regulator can be found that could be a target for an insulin mimetic that would repress all TIRE-containing promoters rather than a select few.

Acknowledgements: This work was supported by Wellcome Trust Career Development Award 051792, and BBSRC CASE awards (S.P., industrial partner Glaxo Smith Kline and C.L., industrial partner AstraZeneca), while C.S. is a recipient of a Diabetes UK Senior Fellowship (RD02/0002473). The work was also supported by generous donations from the DDS Trust, Tenovus Scotland, and the Anonymous Trust, Scotland.

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